Environmental impacts of an imidacloprid-containing formulation: from soils to waters

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Impactos ambientales de una formulación que contiene imidacloprid: de los suelos a las aguas

Impactes ambientals d'una formulació que conté imidacloprid: dels sòls a les aigües

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RESUMEN

El pesticida neonicotinoide imidacloprid se encuentra entre los agroquímicos más vendidos en todo el mundo. Debido a su amplio uso en mezclas con diferentes disolventes y co-adyuvantes, estudiar el impacto ambiental de las formulaciones comerciales derivadas se ha convertido en obligatorio. En este estudio se utilizaron ensayos ecotoxicológicos de laboratorio para cuantificar el impacto del Confidor® 20SL (formulación que contiene imidacloprid) en los compartimentos terrestre y acuático. Los efectos letales y subletales de las dosis recomendadas de aplicación del producto fueron evaluadas en los invertebrados terrestres *Eisenia foetida* y *Folsomia candida* mientras que la toxicidad de los lixiviados de los suelos contaminados se evaluó en los organismos acuáticos modelo *Daphnia magna* y *Raphidocelis subcapitata* (anteriormente *Selenastrum capricornutum*). La exposición a concentraciones ambientalmente relevantes de imidacloprid no causó mortalidad en las lombrices de tierra (CL_{50} de 4.23 mg de imidacloprid por kg de suelo seco) pero alteró los patrones de comportamiento y reproducción (valores de CE_{50} de 0.43 y 1.40 mg de imidacloprid por kg de suelo seco en los ensayos de alejamiento y reproducción respectivamente). Los efectos en los colémbolos *F. candida* fueron despreciables. El imidacloprid presentó una lixiviabilidad moderada, con tasas de recuperación en los extractos acuosos que fueron del 25.4 al 50.4% de la cantidad presente en los suelos y concentraciones de 13.05 a 71.8 µg por litro. Las pruebas estándar de ecotoxicidad acuática no fueron capaces de detectar toxicidad aguda o crónica en los organismos de ensayo. Sin embargo, las concentraciones de insecticida en los extractos fueron lo suficientemente grandes como para representar una amenaza letal para otros organismos acuáticos no estándar.

Palabras clave: Imidacloprid; ecotoxicidad; extractos acuosos; lombrices de tierra.

RESUM

El pesticida neonicotinoide imidacloprid es troba entre els agroquímics més venuts a tot el món. Degut al seu ampli ús en mescles amb diferents dissolvents i co-adjuvants, estudiar l'impacte ambiental de les formulacions comercials que en deriven ha esdevingut obligatori. En aquest estudi es van utilitzar assajos ecotoxicològics de laboratori per a quantificar l'impacte del Confidor® 20SL (formulació que conté imidacloprid) en els compartiments terrestre i aquàtic. Els efectes letals i subletals de les dosis recomanades d'aplicació del producte van ser avaluades en els invertebrats terrestres *Eisenia foetida* i *Folsomia candida* mentre que la toxicitat dels lixiviats dels sòls contaminats es va avaluar en els organismes aquàtics model *Daphnia magna* i *Raphidocelis subcapitata* (anteriorment *Selenastrum capricornutum*). L'exposició a concentracions ambientalment rellevants d'imidacloprid no va causar mortalitat en els cucs de terra (CL_{50} de 4.23 mg d'imidacloprid per kg de sòl sec) però en va alterar els patrons de comportament i reproducció (valors de CE_{50} de 0.43 i 1.40 mg d'imidacloprid per kg de sòl sec en els assajos d'allunyament i reproducció respectivament). Els efectes en els col·lèmbols *F. candida* van ser menyspreables. L'imidacloprid va presentar una lixiviabilitat moderada, amb taxes de recuperació en els extractes aquosos que van anar del 25.4 al 50.4% de la quantitat present en el sòls i concentracions de 13.05 a 71.8 µg per litre. Les proves estàndard d'ecotoxicitat aquàtica no van ser capaces de detectar toxicitat aguda o crònica ens els organismes d'assaig. No obstant això, les concentracions d'insecticida en els extractes van ser prou grans com per a representar una amenaça letal per a altres organismes aquàtics no estàndard.

Paraules clau: Imidacloprid; ecotoxicitat; extractes aquosos; cucs de terra

SUMMARY

The neonicotinoid pesticide imidacloprid is among the top sold agrochemicals worldwide. Due to its widespread use in mixtures with different solvents and co-adjuvants, studying the environmental impact of its derived commercial formulations has become mandatory. In this study we used laboratory ecotoxicological tests to quantify the impact of the imidacloprid-containing formulation Confidor®

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20SL on the terrestrial and aquatic compartments. Lethal and sublethal effects of recommended application doses of the product were assessed on standard terrestrial invertebrates *Eisenia fetida* and *Folsomia candida* whereas the toxicity of leachates from contaminated soils was evaluated in the aquatic model organisms *Daphnia magna* and *Raphidocelis subcapitata*. The exposure to environmentally relevant concentrations of imidacloprid caused no mortality to earthworms (LC_{50} of 4.23 mg imidacloprid kg^{-1} dry soil) but altered their behavior and reproduction patterns (EC_{50} values for avoidance and reproduction tests of 0.43 and 1.40 mg imidacloprid kg^{-1} dry soil, respectively). Effects on collembolans *F. candida* were negligible. Imidacloprid presented moderate leachability, with recovery rates that ranged from 25.4 to 50.4% of the amount present in soils and concentrations in water extracts from 13.05 to 71.8 µg L^{-1}. Standard aquatic ecotoxicity tests were not able detect chronic or acute toxicity in standard test organisms. Nonetheless, concentrations of the insecticide in water extracts were high enough to pose a lethal threat to several other non-standard aquatic organisms.

Keywords: *Imidacloprid, ecotoxicity, water-extracts, earthworms*

1. INTRODUCTION

Despite the potential harmful effects of pesticides, the massive application of plant protection products is necessary in order to provide enough food to satisfy the demands of an increasing human population. Neonicotinoids are a relatively new group of systemic insecticides developed in the 1980s and first commercially available in the form of imidacloprid since early 1990s (Kollmeyer et al. 1999). They bind to the post-synaptic nicotinic acetylcholine receptors (nAChRs) in the central nervous system of insects, thereby disrupting their nerve impulses. Due to their systemic activity, high toxicity to insects, low toxicity to vertebrates and versatile application, neonicotinoids are among the largest selling and most used pesticides worldwide (Elbert et al. 2008; Jeschke et al. 2011; Main et al. 2014). Within this group of insecticides, imidacloprid-containing formulations account for up to 41% of the neonicotinoids market, becoming the second most used agrochemical worldwide (Jeschke et al. 2011; Pollack 2011).

The prophylactic use of imidacloprid during the last decades has led to serious environmental concerns because of its chemical properties. Regardless of the application route of imidacloprid-containing formulations, the bulk of the active ingredient ends up in soil, where it is subjected to various transformation and transportation processes. Due to its high persistence because of a generally long half-live in soils, non-target soil organisms and terrestrial pollinators are usually exposed to fluctuating concentrations of the insecticide. During the last decades, detrimental effects after exposure to imidacloprid have been documented in terrestrial snails (Radwan and Mohamed. 2013), beetles (Russell et al. 2010), earthworms (Luo et al. 1999; Capowiez et al. 2003; Dittbrenner et al. 2010; Dittbrenner et al. 2011), collembolans (Idinger 2002; Alves et al. 2014) and bees (Decourtye et al. 2004; Dively et al. 2015) among others. Furthermore, its high water solubility, high partitioning and low soil sorption enhance the movement of the neonicotinoid from the terrestrial to the aquatic compartment by spray drift, leaching or surface runoff (Roessink et al. 2013). Concentrations of imidacloprid have been measured in surface and ground waters worldwide (Lamers et al. 2011; Starner and Goh 2013) and toxic effects have been documented in many aquatic non-target organisms (Tisler et al. 2009; LeBlanc et al. 2012, Roessink et al. 2013; Pérez-Iglesias et al. 2014 among others).

In the European Union, ecotoxicological laboratory tests are used as a preliminary step in the assessment of the environmental impacts of pesticides and are required prior to the sale of plant protection products (EC 2009). Most laboratory tests follow standardized guidelines to study the toxic effects that pesticides cause to a set of non target model organisms that play key roles in ecosystem structure and function. Among the invertebrate species mostly recommended for terrestrial ecotoxicological assays, acute and chronic effects of imidacloprid have been reported in *Eisenia fetida* (Dittbrenner et al. 2011; Alves et al. 2013) and *Folsomia candida* (Idinger 2002; Alves et al. 2014). Similarly, aquatic ecotoxicology have been traditionally applied for the toxicity determination of aquatic pollutants (Lopez-Roldan et al. 2012), industrial effluents (Riva et al. 1993; Riva and Valles 1994; Riva et al. 2007) or elutriates of sediments (Pereira-Miranda et al. 2011) among others. Effects of imidacloprid on the aquatic environment have been mostly studied through standard aquatic toxicity tests with the model organisms *Daphnia magna* (Crustacea) and *Raphidocelis subcapitata* (Chlorophyta) (Pavlic et al. 2005; Jemec et al. 2007; Tisler et al. 2009; Malev et al. 2012). Unfortunately, the application of ecotoxicity tests for the regulation of pesticides have traditionally focused on parental compounds, passing over the fact that are commercial formulations instead of pure active ingredients the ones applied in the environment. This approach neglects the effects of some co-formulants and solvents present in commercial formulations that can be more important than the active substances to non-target organisms (Anderson and Roberts 1983; Neves et al. 2001) due to its own toxicity or through the modification of the toxicity and bioavailability of the pesticide (Malev et al. 2012). Furthermore, it is known that the leaching potential of pesticides is affected by the type of formulation, surfactants and adjuvants (Camazano et al. 1995; Hall et al. 1998).

Despite the amount of available data regarding the impacts of imidacloprid to non-target organisms, data on the toxicity of imidacloprid-containing formulations is scarcer. Data on such commercial products is required since some studies revealed a higher toxicity and leaching potential of the commercial formulation in comparison with the active ingredient (Gupta et al. 2002; Jemec et al. 2007; Malev et al 2012). In order to widen the available information on this formulation, we studied the environmental impacts associated to the field application rates of Confidor® 20SL.

Table 1. *Physical-chemical parameters of the test soil. C/N: carbon-nitrogen ratio; CEC: cation exchange capacity*

Moisture (%)	pH	Organic carbon (%)	Organic matter (%)	Total nitrogen (%)	C/N	$N\text{-}NO_3$ (mg/kg)	CEC meq/100g	Textural class
3.0	7.2	6.2	10.7	0.4	16.9	15	22.8	Loamy

Effects on the terrestrial compartment were assessed through standard ecotoxicity tests that evaluated the mortality, inhibition of reproduction and avoidance behavior of earthworms *E. fetida* and avoidance of collembolans *F. candida* after exposure to treated soils. Impacts on the aquatic compartment were assessed through the leaching of treated soils and the evaluation of the acute effects of the water extracts to the non-target aquatic invertebrate *D. magna* and the microalgae *R. subcapitata*. Following this methodology, the main objective of this study was to characterize via lower-tier standard ecotoxicological tests the risk that the application of the recommended field rates of the commercial formulation Confidor® 20SL poses to the aquatic and terrestrial compartments.

2. MATERIALS AND METHODS

A soil from a known natural uncontaminated area near the laboratory was selected for the performance of the tests. Samples were collected from the topsoil (0-20 cm depth), air-dried and sieved through a 2 mm mesh. Several soil parameters were analyzed: moisture, pH, organic carbon, organic matter, total nitrogen, C/N ratio, N-NO_3, cation exchange capacity and texture (Table 1).

The insecticide Confidor® 20SL (soluble concentrate, 20% imidacloprid (w/v)) was purchased from Bayer (Germany). Toxicity tests were performed in a range of concentrations that included the lowest and highest application rates recommended by the manufacturer (0.5 and 4 L Confidor ha^{-1}, respectively), two intermediate concentrations (1 and 2 L Confidor ha^{-1}) and a concentration of 8 L Confidor ha^{-1} to cover the worst case scenario of an excessive application of the insecticide. Assuming a depth of incorporation in the soil profile of 0-5 cm and a density of 1.5 g/cm^3, the application rates of Confidor amounted to 0.78-1.56-3.1-6.20-12.4 mg per kg of soil dry weight (dw) and corresponded to 0.13-0.26-0.5-1-2 mg of imidacloprid kg^{-1} dry soil respectively. The application of the formulation into the soil consisted in preparing a stock solution of 1000 mg Confidor L^{-1} in deionized water. Different spiking solutions were applied to the soil in order to provide the desired concentrations of test substance and a moisture content of 60% of the WHC. Soils were carefully mixed to ensure an evenly distribution of the pesticide and left overnight for equilibration. Only deionized water was added to the controls.

Water-extracts were obtained from each soil following the British Standard EN 12457-2 (2002). Soil samples were incorporated to 2-L glass vessels at a ratio of 1 kg/10 L, corresponding to 0.1 kg of soil per liter of deionized water. Vessels were placed at a rotating apparatus and mixed during 24 hours at a temperature of 20±2ºC. After a settling period of 15 minutes, samples were centrifuged (2000g, 10 minutes) and filtered. The supernatant was kept refrigerated until use. The concentration of imidacloprid in the leachates was analyzed by SAILab (Cerdanyola del Vallès, Barcelona, Spain) by High Performance Liquid Chromatography/MS (Agilent 1200 LC/ Applied Biosystems 3200 LMS).

Synchronized cultures of earthworms *E. fetida* and collembolans *F. candida* were obtained from the Centre for Research and Innovation in Toxicology of the Technical University of Catalonia (UPC) in Terrassa (Spain). Earthworms were bred in a cow manure–peat mix (1:1, w/w) at a temperature of 20±2 ºC and under a 16:8 light:dark photoperiod and were fed once a week with moistened bread. Forty-eight hours prior to starting the tests, adult clitellate animals were acclimated to the untreated soil. Only individuals weighting between 300 and 600 mg were selected. Collembolans were cultured in vessels filled with a substrate of plaster of Paris and charcoal (8:1 w/w) at 20±2ºC. Individuals were fed twice a week with granulated dry yeast added in small amounts to avoid spoilage by fungi. Organisms between 10 and 20 days old were selected for avoidance tests. Terrestrial bioassays were performed in a climate-controlled room at 20±2ºC and under a 16:8 light-dark photoperiod except for the acute toxicity test with earthworms that was carried out under constant illumination (400-800 lux).

Lethal effects to *E. fetida* were assessed following the recommendations by the OECD guideline 207 (OECD 1984). Ten individuals were placed in plastic containers containing 500 g of spiked soil (dw). Four replicates were prepared per test concentration. The percentage of mortality and pathological symptoms were monitored after 7 and 14 days of exposure. As no mortality was expected at field application rates of the pesticide, higher concentrations of Confidor were included in order to estimate the LC_{50}.

Effects on the reproduction of earthworms were studied by means of the OECD 222 (2004) guideline. Ten earthworms were placed in 1-L plastic containers filled with 500 grams of dry soil. Four replicates per test concentration and 6 replicates for the control were prepared. Animals were fed weekly with 2 grams of moistened bread during 4 weeks. After 28 days of exposure, surviving earthworms were sorted by hand and the mortality and changes in biomass were recorded. Juvenile worms and cocoons remained in the test vessels for another 28 days. The number of juveniles was recorded after 56 days by heating the soils in a warm bath at 60ºC for 20-25 minutes and waiting for the juveniles to emerge.

Avoidance tests with *E. foetida* and *F. candida* were carried out according to the ISO 17512 (2008) and ISO 17512 (2011) standards respectively. Tests were performed in plastic containers divided into two equal sections by a vertically introduced plastic card. In the test with earthworms, each side of the vessel (control and test) was filled with 350g (dw) of the corresponding soil and the divider was removed. Ten adult earthworms were placed in the line separating both soils. In the test with collembolans, 25 g (dw) of soil were filled into the corresponding section and twenty springtails were carefully placed on top of the soils. In both cases tests ran with five replicates per concentration. At the end of the test period the plastic card was reinserted and the number of individuals at each section counted. In tests with collembolans, the soil from each section was carefully emptied into two different vessels and flooded with water. After gentle stirring the animals floating on the water surface were counted. Missing animals were considered as dead organisms and discarded for the later calculations. Dual-control tests were carried out with both methodologies (5 replicates each) to guarantee the homogeneous distribution of the organisms in the absence of the test substance.

Toxicity in the aquatic compartment was tested in two model species, the cladocera *D. magna* and the microalgae *R. subcapitata*. Cultures of 15 daphnids were maintained in 2.5 L ASTM hard synthetic water kept at 20±2ºC in a 16:8h light:dark cycle. Culture media were changed

three times per week and an organic extract and a concentrate of *Chlorella vulgaris* were added as food. Neonates were collected daily and only those less than 24 hours old were used in tests. Cultures of the algae *R. subcapitata* were kept under a constant illumination of 4000-5000 lux at 20±2ºC. Only populations in the exponential phase were used for the assays. The acute toxicity test with *D. magna* was carried out according to the OECD Guideline 202 (1984). Four replicates were prepared per leachate. Each replicate consisted in a glass tube with 10 mL of the corresponding leachate and 5 daphnids. The test was performed in an incubator at 21ºC and in the dark. Immobilization was visually recorded after 24 and 48 hours of exposure. Chronic toxicity to *D. magna* was evaluated following the OECD Guideline 211 (1998) for a semi static exposure system. Ten replicates per leachate were prepared, each consisting of a 250 mL glass vessel filled with 75 mL of test solution and one daphnid. During the assay, test solutions were replaced and enriched with seaweed extract three times per week. Animals were fed with a concentrate of *Chlorella vulgaris* (0.1-0.2 mg per day). The assay was carried out in a controlled room for 21 days at a temperature of 20±2ºC and a light:dark cycle of 16:8 hours. The growth inhibition test with *R. subcapitata* was carried out following the recommendations of the OECD Guideline 201 (1984). The test ran with 3 replicates for each water extract from contaminated soils plus the leachate from the control soil and an additional control with algae culture medium. Each replicate consisted in 9 mL of test solution and 1 mL of algal inoculum of known concentration. In order to avoid interferences in the spectrometric measure of the leachates at the end of the test, one extra tube was prepared with 9 mL of leachate, 1 mL of culture medium and no algae. The tubes were placed in a controlled room at 20±2 ºC under constant light (4000-5000 lux) and agitation. After 72 hours of incubation, the absorbance of each replicate was measured at 665 nm with a CECIL CE9200 spectrophotometer in order to determine the final algal concentration.

Results of toxicity tests were calculated as percentages. Differences between treatment means (i.e., different concentrations of Confidor) were tested through Analysis of Variance (ANOVA)(P<0.05). When significant differences were detected, the Dunnet post-hoc test was applied to compare treatment means with the control using SPSS 19.0 (NY, USA) software. NOEC (No observed effect concentration) and LOEC (Lowest observed effect concentration) values were established through this procedure. The percentage of avoidance was calculated following the equation presented in the ISO standards 17512 (2008) and 17512 (2011):

$$x = \left(\frac{n_c - n_t}{N}\right) \times 100$$

where x is avoidance, expressed as a percentage; n_c is the number of individuals in the control soil; n_t is the number of individuals in the test soil and N is the total number of individuals. The significance of the avoidance responses were analyzed using the Fisher Exact test (Zar 1998). A two-tailed test were used in the analysis of the dual-control test and a one-tailed test was used for the polluted soils. The null hypothesis assumed an even distribution of individuals between both soil sections and was rejected for a probability equal or lower than 0.05. Median lethal concentration (LC_{50}) values and effective median concentration values (EC_{50}) were estimated by the Probit method following logistic regressions with Statistica software version 8.0 (OK, USA) and Minitab 13.20 software (PA, USA) respectively.

3. RESULTS AND DISCUSSION

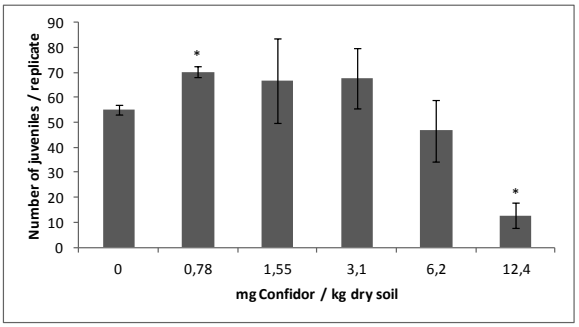
The exposure of soil invertebrates to field doses of Confidor in standard ecotoxicity tests showed marked differences in sensitivity between endpoints and test species. Mortality of earthworms occurred at concentrations higher than 19.77 mg Confidor kg^{-1} (soil dw) (LOEC) (Table 2) and the LC_{50} was estimated at 24.71 mg kg^{-1} dry soil (corresponding to 4.23 mg imidacoprid kg^{-1} dry soil), indicating that the recommended doses of the formulation did not represent a lethal threat to *E. fetida.* Similar toxicity values were reported by Luo et al. (1999) and Gomez-Eyles et al. (2009) using pure imidacloprid as test substance (LC_{50} values of 2.30 mg kg^{-1} soil dw and 2.36 mg kg^{-1} soil dw respectively). On the other hand, studies by Kreutzweiser et al. (2008) and Alves et al. (2013) reported LC_{50} values 10 times higher (25 and 25.53 mg imidacloprid kg^{-1} soil dw respectively) after applying the commercial imidacloprid-containing formulations Merit Solupak® and Gaucho®. Differences in LC_{50} values between studies were partly explained by variations in experimental parameters like soil organic matter, texture or time of exposure (Kula and Larink 1997) although the influence of certain components from commercial formulations to the overall toxicity of the product was not discarded.

Table 2: *EC_{50} (effect concentration 50%), LC_{50} (lethal concentration 50%), confidence intervals (95%), LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) values of Confidor / imidacloprid estimated for earthworm mortality, reproduction and avoidance tests. Values presented in [mg Confidor /kg soil dw] / [mg Imidacloprid /kg soil dw]*

Test	EC_{50}(LC_{50})	Lower limit (95%)	Upper limit (95%)	LOEC	NOEC
Mortality	24.71/4.23	23.30/3.99	26.20/4.48	19.77/3.38	15.21/2.6
Reproduction	8.41/1.40	5.38/0.90	12.87/2.15	12.40/2	6.20/1
Avoidance	2.57/0.43	1.86/0.31	3.21/0.54	0.78/0.13	<0.78/<0.13

The reproduction test gave varying results depending on the concentration of pesticide in soil. *E fetida* produced a significantly higher number of juveniles (Dunnet's test, P < 0.05) in soils treated with the lowest application rate of imidacloprid than in untreated soils (Fig. 1). On the other hand, significant detrimental effects on the reproductive output occurred at twice the highest recommended dose (12.4 mg Confidor kg^{-1} soil dw)(LOEC). The EC_{50} for the reproduction test was estimated at 8.41 mg Confidor kg^{-1} soil dw (corresponding to 1.40 mg imidacloprid kg^{-1} soil dw) (Table 2), a concentration that could be easily reached if the formulation is not properly employed in terms of applied concentrations or time between applications. A similar EC_{50} value (1.41 mg kg^{-1} soil dw) was reported by Gomez-Eyles et al. (2009) using pure imida-

cloprid as test substance whereas a study by Alves et al. (2013) observed a significantly lower toxicity (EC_{50} value of 4.07 mg imidacloprid kg^{-1} soil dw) of a imidacloprid-containing formulation. Luo et al. (1999) and Capowiez and Berard (2006) linked the decrease in the reproductive output to the damage exerted by imidacloprid to spermatozoa of earthworms. It was not concluded whether differences in toxicity between studies were due to the experimental conditions or to the nature of the test substance (active ingredient or commercial formulation). Additionally, it is noteworthy the hormetic response that Confidor triggered in the reproductive output of exposed earthworms. An enhanced reproduction rate was previously documented by Senapati et al. (1992) and Suthar (2014) after exposing earthworms to low concentrations of the pesticides malathion and methyl parathion respectively although the biochemical mechanism of this response is not clear yet. Similar results have not been reported for other neonicotinoids or neonicotionid-based formulations. Regarding the reduction of body weight, it followed the same pattern than juvenile production, with an average weight loss lower than controls at low application rates and significantly higher at high test concentrations (Fig. 1).



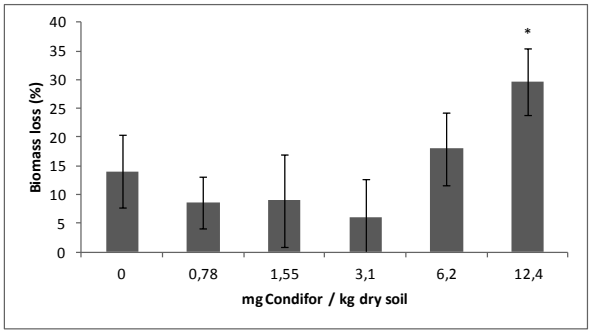


Figure 1: *Effects of varying concentrations of Confidor on the reproductive output and weight loss of E. Fetida in reproduction tests. Data presented as treatment means ± SD(N=4). Asterisks indicate significant differences with controls (Dunnet's test, P < 0.05).*

Earthworms exhibited a significant avoidance behavior in response to the presence of all test concentrations of the formulation (Figure 2). The LOEC value was established at the lowest tested concentration, corresponding to the minimum application rate recommended by the manufacturer (Table 2). Furthermore, the EC_{50} value was estimated at 2.57 mg Confidor kg^{-1} soil dw, within the range of recommended doses. According to Hund-Rinke and Wiechering (2001), soils contaminated with concentrations of Confidor higher than 1.56 mg kg^{-1} soil dw presented a reduced habitat function and should be considered as toxic to earthworms since they presented avoidance responses higher than 60% (i.e more than 80% of individuals remained at the control section of the test chamber). Our results were in accordance with those from Alves et al. (2013) who estimated an EC_{50} value of 0.11 mg kg^{-1} in *Eisenia andrei* for a commercial formulation of imidacloprid. In contrast, Capowiez and Bérard (2006) reported no avoidance response of earthworm species *Aporrectodea nocturna* and *Allolobophora icterica* after exposure to 0.5 and 1 mg kg^{-1} (soil dw) of Confidor® 200 SL despite previous studies documented behavioral alterations on burrow length, overall distance travelled and rate of burrow reuse under the same experimental conditions (Capowiez et al. 2003). Similarly, earthworms exposed to the pesticide in our study presented an altered locomotion pattern. After the increase in the avoidance response observed at 0.78 and 1.56 mg Confidor kg^{-1} soil dw, the behavioral response turned stable while increasing test concentrations. A study by Pereira et al. (2010) reported that the exposure of *E. Andrei* to the carbamate insecticide methomyl induced a inhibition of the Acetylcholine esterase activity that led to hyperactivity in the test organisms and in consequence to the adoption of an irregular avoidance behavior. Similar conclusions were postulated by Martínez Morcillo et al. (2013) after exposing earthworms from the species *Lumbricus terrestris* to chlorpyrifos, another insecticide known to affect the nervous system of soil invertebrates. Based on behavioral alterations reported by Capowiez et al. (2003) and the mechanism of action of imidacloprid, we hypothesized that the exceeding of certain toxicity threshold somehow altered the locomotive ability of the test organisms and led to an erratic movement pattern, thus causing the stabilization of the avoidance response. In the case of collembolans, an avoidance behavior in response to the application of Confidor recommended doses was not detected at any test concentration. Furthermore, a significant preference for the contaminated soil (Fisher exact test, $P < 0.05$) was observed at concentrations of 3.1 and 12.4 mg Confidor/kg dw (data not shown).

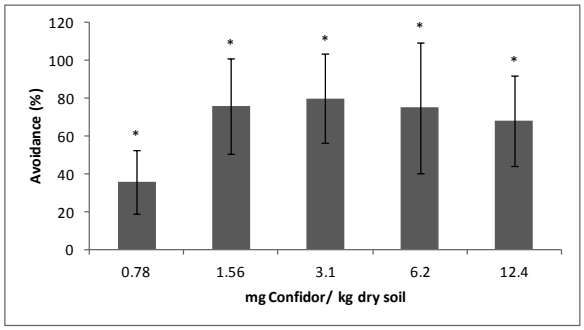


Figure 2: *Avoidance response (%) of E. fetida (mean ± SD)(N=5) to varying concentrations of Confidor in avoidance tests. Asterisks indicate significant differences with the control (Fisher's test, P < 0.05).*

To determine the leaching potential of imidacloprid and its risk for aquatic organisms, concentrations of imidacloprid were determined in water extracts from contaminated soils (Table 3). The concentrations of active ingredient in leachates ranged from 13.05 µg L^{-1} (corresponding to the soil treated with 0.26 mg imidacloprid kg^{-1} dw) to 71.8 µg L^{-1} (2 mg imidacloprid kg^{-1} soil dw) and were positively correlated with concentrations in test soils ($r = 0.910$, $P < 0.05$,

Spearman). The concentrations of imidacloprid in water extracts were within the range estimated by Fossen (2006) for chronic and acute surface water exposures (17.24 and 36.04 µg L^{-1} respectively) or after accidental direct spray in a pond or stream (22 µg L^{-1})(SERA 2005). The recovery of the pesticide ranged from 25.4% to 50.4% of the total amount previously spiked in soil. Recovery rates were in accordance with the relatively high water solubility (0.5 to 0.6 g L^{-1}) and low octanol-water partitioning coefficient (Log (Pow)=0.57) of imidacloprid reported by other authors (Gupta et al. 2002; Kurdwadkar et al. 2014) but were higher than expected according to the high organic carbon content of our soil, a parameter positively correlated with imidacoprid sorption in soils (Cox et al. 1998).

Table 3: *Concentration of imidacloprid in water extracts from contaminated soils. Means ± Standard deviations (N=3).*

mg Confidor / kg soil (dw)	mg imidacloprid / kg soil (dw)	Water extract (µg/L leachate)	Recovery rates (%)
0.78	0.13	< QL	-
1.56	0.26	13.05±3.04	50.35±11.95
3.1	0.5	16.35±4.60	32.70±9.19
6.2	1	25.4±8.21	25.4±8.21
12.4	2	71.8±0	35.9±0

QL (quantification limit): 1 µg/L

Although the highest concentration of imidacloprid determined in water extracts was almost 10^3 times lower than LC_{50} values found in bibliography for *D. magna* (85 mg L^{-1}) (Fossen 2006), mortality tests were performed since previous studies reported the higher toxicity of imidacloprid-containing commercial formulations to *D. magna* due to the presence of toxic adjuvants (Jemec et al. 2007). The exposure to the leachates caused no mortality after 48 hours of exposure in the acute toxicity test and 21 days in the reproduction test. Similarly, differences with the control in the number of neonates per adult, brood size, day of first brood and number of broods per adult in the chronic test were not detected (LOEC value in chronic tests estimated between 2.5 and 10 mg L^{-1} (Jemec et al. 2007)). Regarding the effects on the microalgae *R. subcapitata,* algal growth rates in water extracts from all soils (including the untreated soil) were significantly lower than in algal culture medium (data not shown). However, no significant differences in growth inhibition were found between soil leachates. Consequently, algal growth inhibition was related to the fact that water parameters deviated from the standard test medium and not to the presence of the insecticide in soil leachates. Results with this model organism were expected based on the insecticidal type of action of imidacloprid and its estimated EC_{50} values (> 600 mg L^{-1})(Daam et al. 2013) although previous studies reported the high toxicity to algae of some Confidor® 200 SL co-formulants (Malev et al. 2012). We hypothesized that the lower toxicity detected in our study was related to the fact that in previous studies the commercial formulation was directly spiked into water while we used leachates from contaminated soils. Since the purpose of adjuvants is associated to the fixation of the pesticide in soil, we expected a lower leachability of potentially toxic co-adjuvants.

Despite the low toxicity of leachate concentrations of imidacloprid to the standard organisms *D. magna* and *R. subcapitata*, the presence of the active ingredient in the water extracts was high enough to represent a lethal or sublethal threat to several other non-standard, freshwater macroinvertebrate species. Based on the available bibliography, Daam et al. (2013) reported that a concentration of 52 µg of imidacloprid L^{-1} (value that could be easily reached in soils if Confidor is improperly applied) was expected to produce 50% affection to 25% and 79% of the crustacean and insect taxa respectively. Furthermore, Roessink et al. (2013) documented LC_{50} and EC_{50} values for the non-standard insect species *Notonecta* spp., *Micronecta* spp*., Limnephilidae*, *Caenis horaria* and *Cloeon dipterum* and the macrocustacean *Gammarus pulex* close or below 25 µg imidacloprid L^{-1} , a concentration of active ingredient reached in our leachates.

4. CONCLUSION

Our study pointed out that the application of recommended field doses of the imidacloprid-containing formulation Confidor® 20SL represents a potential threat for the environment. Although mortality was not reported, the exposure to the pesticide caused sublethal effects to *E. fetida* earthworms. The influence of some co-adjuvant and solvents to the overall toxicity of pesticide formulations was observed after comparing results from terrestrial ecotoxicity tests with imidacloprid with those from commercial products. Confidor presented toxicity levels in terrestrial standard ecotoxicity tests closer to those from the active ingredient than to other commercial formulations. Additionally, reproduction and avoidance tests with earthworms showed responses that had not been previously reported, highlighting the need to keep studying the impacts of massively-applied pesticides.

The application of Confidor® 20SL to agricultural soils posed a risk to the aquatic compartment due to the high leachability of imidacloprid. Despite the low response of aquatic standard ecotoxicity tests to the presence of the pesticide or to other components of the formulation, final concentrations of the insecticide in the aquatic compartment were high enough to represent a lethal threat to many other non-standard, non-target aquatic organisms, thus emphasizing the need for testing organisms from different taxonomical groups when assessing the environmental risks posed by pesticides.

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